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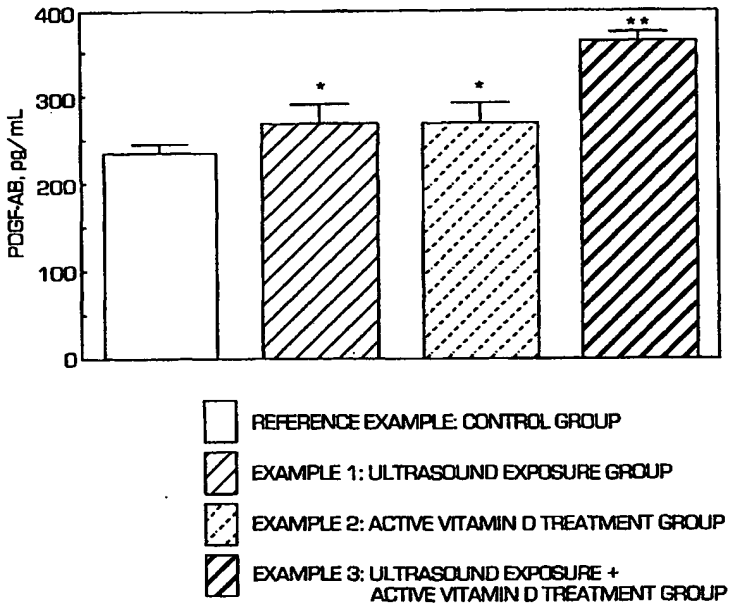
(54) Title: A METHOD FOR INDUCING PRODUCTION OF GROWTH FACTORS



(57) Abstract

A method of enhancement of growth factors including PDGF, which enables them to be used in the treatment of bone fractures and wounds in the future. The more efficient production of growth factors in this invention can be applied as a therapeutic agent for fracture and/or wound healing.

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A METHOD FOR INDUCING PRODUCTION OF GROWTH FACTORS

FIELD OF THE INVENTION

This invention concerns a method for enhancing production of growth factor in coculture systems. More specifically the invention relates to a method of production of growth factor, including platelet derived growth factor (hereinafter referred to as PDGF).

BACKGROUND OF THE INVENTION

Concerning methods for enhancing production of growth factors, it was reported that PDGF production was induced by adding active vitamin D to the culture medium of human keratinocytes by Zang et al., *J Dermatol* 22, 305-309, (1995) in a single culture system. Methods for enhancing production of PDGF in coculture systems are not known at present. And a more efficient method for enhancing production of growth factors is needed.

In view of the above, a series of assiduous studies were conducted to provide a more efficient method for enhancing production of growth factors in coculture systems.

SUMMARY OF THE INVENTION

This invention provides embodiments comprising methods for enhancing production of growth factor in a coculture system by (1) adding an active form of vitamin D to a coculture medium, (2) exposing the cells in a coculture medium to ultrasound and (3) adding an active form of vitamin D to a coculture medium and exposing the cells in the coculture medium to ultrasound. The invention is described below in detail.

BRIEF DESCRIPTION OF THE DRAWING

The Figure shows the amounts of PDGF-AB in the coculture medium wherein:

a) Control group

b) Ultrasound exposure group

c) Active vitamin D treatment group

d) Ultrasound exposure + active vitamin D treatment group

The asterisk (*) and the double asterisk (**) indicate a P value of <0.05 and <0.01, respectively, compared with the control group according to the results of the Dunnett's multiple comparison test.

DETAILED DESCRIPTION OF THE INVENTION

The invention is applicable to growth factors such as basic fibroblast growth factor (bFGF), transforming growth factor-ß (TGF-ß), and PDGF. Among them, PDGF is preferred.

These growth factors have been suggested to play important roles in the tissue repair process, e.g., in fracture repair [Joyce et al. *Prog. Clin. Biol. Res.* 365, 391-416, (1991)]. One of them, PDGF has been found to participate in extremely important vital phenomena in body development, cell differentiation, cell proliferation, etc. In addition, PDGF possesses growth stimulating activity toward almost all mesodermally derived cells. Because PDGF possesses such activities, it has been expected to be used as a therapeutic agent, such as a neovascularization promoting agent or a wound healing agent. Actually, it has been found to play an important role in wound healing process [H. Hosgood, *Vet Surg* 22, 490-495 (1993), United States Patent No. 5,457,093]. In addition, fracture repair can be accelerated by its local injection, and it can be used as a therapeutic agent for fracture.

Coculture systems of the invention are the culture of two or more kinds of cells which produce growth factors, in the same medium. Among them, the combination of osteoblasts and endothelial cells is preferred. Osteoblasts as described herein mean human osteosarcoma cell (SaOS-2), HOS, M063, etc. These cells can be obtained from the American Type Culture Collection, and experimentally used by culturing, and subculturing as described below.

Endothelial cells as described herein means human umbilical vein endothelial cells (RUVEC), microvascular endothelial cells, pulmonary endothelial cells, etc. These cells can be obtained from Sanko Junyaku Co. Ltd., Takara Co. Ltd., and Iwaki Glass Co. Ltd., of Japan etc. These cells can be used by culturing, and subculturing in a medium for endothelial cells. The coculture system of osteoblasts and endothelial cells is established by culturing

osteoblasts in the culture medium as described below, and adding endothelial cells onto osteoblasts the day after resulting in a coculture system.

Culture media used in the invention for the coculture system mean McCoy's 5A modified medium, a-MEM containing 5-15% fetal calf serum, etc. Among them, McCoy's 5A modified medium containing 10 % fetal calf serum is preferred.

The active form of vitamin D used in the invention means a form of vitamin D that possesses physiological activities, such as calcium and bone metabolism regulating activities, as distinguished from forms of vitamin D that have no physiological activity, and includes active forms of vitamin D_2, active forms of vitamin D_3, and their derivatives. Actual examples include 1-hydroxyvitamin D, 1,24-dihydroxyvitamin D, 1,25-dihydroxyvitamin D, 1,24,25-trihydroxyvitamin D, 24,24-difluoro-1,25-dihydroxyvitamin D, and 26,26,26,27,27,27-hexafluoro-1,25-dihydroxyvitamin D. Among them, 1-hydroxyvitamin D_3, 1,24(R)-dihydroxyvitamin D_3, and 1,25-dihydroxyvitamin D_3 are preferred. Concentrations which can be added to the culture medium are $1x10^{-10}$M to $1x10^{-7}$M, and the range of concentration of $1x10^{-5}$M to $3x10^{-8}$M is preferable.

In a preferred embodiment low-intensity ultrasound is that which is not associated with thermal events. Low-intensity ultrasound consists of a frequency of 1.3 to 2 MHz, a repeat cycle of 100 to 1,000 Hz, a burst width of 10 to 2000 μs, and an intensity up to 100 mW/cm^2. It is further contemplated that other ultrasound energy can be used either with or without thermal effect.

Exposing the undersurface of the wells containing the coculture to ultrasound using an Exogen Co. ultrasound output for 20 minutes a day for 4 consecutive days is preferable.

The characteristics of the ultrasound output employed below were a pulse burst of 200 μs, a frequency of 1.5 MHz, a 1kHz repeat cycle, and the intensity was $30mW/cm^2$.

The ultrasound machine used in these experiments is described in United States Patent (No. 4,530,360) as a basic non-invasive treatment technique and device for applying ultrasound pulses transcutaneously from outside the patient's body close to the affected site.

Low-intensity ultrasound consists of a frequency of 1.3 to 2 MHz, a repeat cycle of 100 to 1,000 Hz, a burst width of 10 to 2000 μs, and an intensity up to 100mW/cm^2. The preferred duration of ultrasound exposure is up to 20 minutes.

In U.S. Patent No. 5,520,612 to Winder et al. preferred ultrasound treatment systems are also disclosed. In those systems low intensity ultrasound at a frequency range of 20 kHz to 10 MHz are employed. The repeat cycle range is 5 to 10 kHz. The duty cycle is from about 5 to 90% and the intensity is up to 100mW/cm^2.

In another United States patent (USP 5,003,965), Talish et al. have described an ultrasound treatment system that consists of a body-applicator-unit connected to a remote control unit by a covered optical cable, and low-intensity ultrasound in this system consists of a frequency of 1.3 to 2 MHz, and an intensity up to 1 to 50mW/cm^2.

The method for enhancing production of growth factors in the coculture system in this invention involves the combination of adding active vitamin D to a coculture medium containing cells producing growth factor, and also to exposing cells to ultrasound is preferable.

EXAMPLES

The following specific examples are given to illustrate the present invention.

Examples 1 to 3 and Comparative Example

A 2-mL volume of human osteoblastic cells (SaOS-2) was seeded at a density of 2x10^5 cells/well into a 6-well plate coated with human type I collagen. SaOS-2 is an osteoblastic cell line that was established in 1975 from a Caucasian female osteosarcoma, and it was obtained from the American Type Culture Collection. McCoy's 5A medium to which 10% fetal calf serum (FCS) had been added (10% FCS - McCoy's 5A) was used as the culture medium. The next day, 0.5 mL of human umbilical vein endothelial cells (HUVECs) was added to each well at a density of 3x10^5 cells/well. The HUVECs were obtained from Sanko Junyaku Co. Ltd.

Each group is shown as below.

Example 1; The undersurface of the wells was exposed to ultrasound for 20 min a day for 4 consecutive days.

Example 2; 10^{-8}M 1,25-dihydroxyvitamin D_3 was added to the culture medium.

Example 3; 10^{-8}M 1,25-dihydroxyvitamin D_3 was added to the culture medium and the undersurface of the wells was exposed to ultrasound for 20 min a day for 4 consecutive days.

In the Comparative Example and Example 1, concerning active vitamin D treatment, the culture medium was replaced with 10% FCS - McCoy's 5A containing 0.01 % ethanol (vehicle for 1,25-dihydroxyvitamin D3). The medium was changed 2 days later.

In Example 2 and Example 3, the culture medium was replaced with 10% FCS - McCoy's 5A containing $1x10^{-8}$M 1,25-dihydroxyvitamin D3. The medium was changed 2 days later.

Concerning ultrasound exposure, in Comparative Example and Example 2, there was no exposure to ultrasound. In Example 1 and Example 3, the exposure to low-intensity ultrasound with an Exogen Co. ultrasound output unit, 20 minutes a day, for 4 consecutive days. The characteristics of the ultrasound output were a pulse width of 200 μs, a frequency of 1.5 MHz, a 1kHz repeat cycle, and the intensity was 30mW/cm^2.

The culture medium was collected the following day, and the concentration of PDGF-AB in the culture medium was measured with an Amersham Co. ELISA kit that specifically measures PDGF-AB. The results obtained are shown in Figure 1.

As clearly shown in Figure 1, the amount of produced PDGF-AB was significantly increased in Example 1, 2 and 3 compared with the Comparative Example. Furthermore, a greater increase in PDGF-AB in Example 3 was observed among the Example groups.

While the invention has been described in detail and with respect to different embodiments, it will be apparent to one skilled in the art that various changes and modifications can be made without departing from the spirit and scope thereof.

WHAT IS CLAIMED IS:

1. A method for enhancing production of growth factor comprising the step of: coculturing cells producing growth factor in the presence of active vitamin D.

2. A method for enhancing production of growth factor comprising the step of coculturing cells producing growth factor while exposing the cells to ultrasound.

3. A method for enhancing production of growth factor comprising the step of culturing cells producing growth factor in the presence of active vitamin D while exposing the cells to ultrasound.

4. A method of claim 1, claim 2 or claim 3 wherein said growth factor is platelet derived growth factor.

5. A method of claim 1, claim 2 or claim 3 wherein the coculturing is of osteoblasts and vascular endothelial cells.

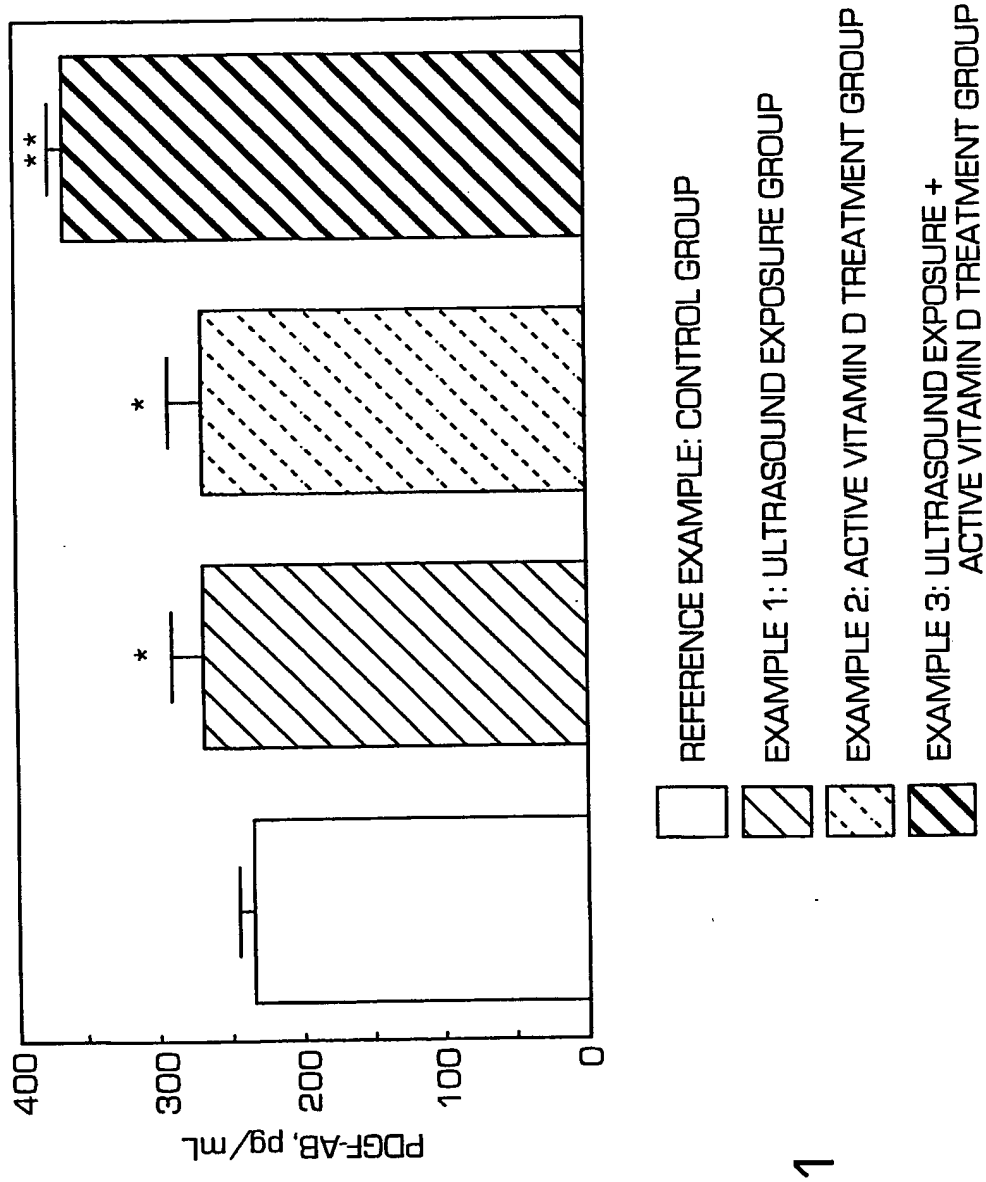
PDGF-AB, pg/mL
400
300
200
100
0
*
*
**
REFERENCE EXAMPLE: CONTROL GROUP
EXAMPLE 1: ULTRASOUND EXPOSURE GROUP
EXAMPLE 2: ACTIVE VITAMIN D TREATMENT GROUP
EXAMPLE 3: ULTRASOUND EXPOSURE + ACTIVE VITAMIN D TREATMENT GROUP

FIG. 1

INTERNATIONAL SEARCH REPORT

Internatic Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/49 C07K14/475 C12N5/06 C12N13/00 C12P21/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WANG ET AL.: "Anabolic effects of 1,25-dihydroxyvitamin D3 on osteoblasts are enhanced by vascular endothelial growth factor produced by osteoblasts and by growth factors produced by endothelial cells" ENDOCRINOLOGY, vol. 138, no. 7, July 1997 (1997-07), pages 2953-2962, XP002118701	1,5
Y	page 2953 -page 2954, column 1; figures 7-9; table 1 page 2957, column 2 -page 2960	2-4
Y	US 5 656 450 A (BOYAN BARBARA D ET AL) 12 August 1997 (1997-08-12) column 12, line 15 - line 33; figure 1 column 12, line 51 - line 56	2-4

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	FUNAYAMA H ET AL: "Human monocyte-endothelial cell interaction induces platelet-derived growth factor expression." CARDIOVASCULAR RESEARCH , vol. 37, no. 1, January 1998 (1998-01), pages 216-224, XP002118702	4
A	page 216 -page 217; figures 1,4	1-3,5
A	ZANG ET AL.: "Production and secretion of platelet-derived growth factor AB by cultured human keratinocytes: regulatory effects of phorbol 12-myristate 13-acetate, etretinate, 1,25-dihydroxyvitamin D3, and several cytokines" JOURNAL OF DERMATOLOGY, vol. 22, no. 5, May 1995 (1995-05), pages 305-309, XP002118703 cited in the application page 305 -page 306, column 1; table 1 page 308, column 1	1-5

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Internatio Application No
PCT/US 99/12815

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5656450 A	12-08-1997	AU 2597595 A	21-12-1995
		CA 2187731 A	07-12-1995
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		JP 10504526 T	06-05-1998
		WO 9532709 A	07-12-1995